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Summary

Using a tritiated (^3H) caffeine tracer and a murine monoclonal anti-caffeine antibody, we developed a radioimmunoassay (RIA) for the detection of caffeine (1,3,7 trimethylxanthine) in saliva. The assay shows <2% cross reactivity with theophylline and avoids interference from anti-mouse immunoglobulin (IgG) constituents found in serum but not in saliva. Saliva caffeine represents the unbound (biologically active) fraction of the drug. Assay values correlate well ($r=.44$, $p<.05$) with oral caffeine doses between 150 and 400 mg. The half-life of salivary caffeine of about six hours, based on the elimination curve for the subjects who received 400 mg, agrees with previous measurements of its half-life in serum.

Introduction

Plasma or serum concentrations of biologically active compounds such as caffeine are the accepted standard for monitoring levels of these compounds in the body. However, obtaining blood samples is an invasive procedure which has potential complications. Trained personnel are required, which can add to the expense of performing nonhospital-based research. In contrast, saliva can be collected noninvasively and can provide point estimates of concentrations of substances that diffuse from plasma to saliva.

Salivary assays have been used to monitor concentrations of hormones which diffuse readily from plasma to saliva, such as thyroxine (Elson, Morley and Shafer, 1983), cortisol and testosterone (Read, Riad-Fahmy, Walker, and Griffiths, 1984; Kirschbaum and Hellhammer, 1989), melatonin (Vakkuri, 1985), estradiol (De-Boever, Kohen, Bouve, Leyseele, and VandeKerchkhove, 1990), and progesterone (Vuorento, Hovatta, Kurunmaki, Ratsula, and Huhtaniemi, 1990). Saliva also has been used for measurements of drug concentrations, including pemoline (Vermeulen, Teunissen and Breimer, 1978), nicotine metabolites (Bjercke, Cook, and Langone, 1987), cyclosporine (Coates, Lam, and McGaw, 1988), ethanol (Ruz, Linares, Luque de Castro, Caridad, and Valcarcel, 1989), anti-convulsants (Miles, et al., 1990), salycilic acid (Cavrini, Gatti, and DiPietra, 1990), and theophyline (Blanchard, Harvey, and Morgan, 1990). Saliva concentrations of these hormones and drugs usually correlate well with the free (nonprotein-bound) fraction in the blood, which is the biologically active form.

Because transfer of methylxanthines from blood into saliva also occurs by passive diffusion, saliva concentrations usually correlate well with concentrations in the blood (Zylber-Katz, Granit, and Levy, 1984). For caffeine, salivary concentrations have been reported to be about 70% of the concentrations in serum

or plasma (Cook et al., 1976; Somani and Khanna, 1981; Walther, Banditt, and Köhler, 1983). Correlations of 95% or higher have been reported between caffeine concentrations in saliva and in serum or plasma (Cook et al., 1976; Khanna et al., 1982) and between the elimination half lives of caffeine from saliva and from serum or plasma (Walther et al., 1983; Zylber-Katz et al., 1984). We present here a new salivary radioimmunoassay (RIA) for caffeine, which is an effective measure under conditions of both repeated and single dose drug administration. We developed this assay because of the recent availability of a monoclonal antibody, of high specificity for caffeine, which allowed the development of a radioimmunoassay that was very sensitive (0.02 μg/ml) and specific (< 2% cross-reactivity with theophylline) and allowed the economy of scale inherent in assaying many samples in a rapid and convenient way.

Methods

Subjects and Procedures

The saliva samples were collected in a double-blind study of the effects of caffeine on naval volunteers during sleep deprivation, conducted at the Naval Health Research Center (NHRC), San Diego, California. Subjects received multiple oral doses of either placebo, 150 mg, 300 mg, or 400 mg of caffeine. There were eight subjects in the placebo group, six in the 150 mg group, eight in the 300 mg group, and eight in the 400 mg group. Capsule administration started at 2330h and continued every six hours for a total of seven capsules. Subjects consumed nothing but water for two hours before and one hour after taking a capsule, and they took nothing by mouth for at least one hour prior to sample collection. The capsules were swallowed with a small amount of water. The 150 and 300 mg groups received active drug at each administration. The

400 mg group received active drug only at the two 2330h administration times, and matched placebo at the other times. Subjects were not permitted to have coffee, tea, caffeinated soft drinks, or chocolate during the study. All subjects were low-to-moderate caffeine users and none used tobacco.

Five ml saliva samples (of which 1.0 ml was used in the RIA) were collected in 16 x 100 mm plastic tubes. The tip of the tongue was touched with lemon juice to stimulate saliva secretion. (Chewing of paraffin or other substances was not used, to avoid possible contamination of the samples.) One sample was collected prior to any caffeine administration, one was collected 40 minutes after each capsule, and one was collected 20 hours after the last capsule. Samples were stored at -20°C and transported from the collection site to the laboratory in dry ice.

Materials

8-[^{3}H] caffeine (Amersham, Arlington Heights, IL), purified by paper chromatography followed by thin-layer chromatography on silica gel, was supplied at a specific activity of 22.2 Ci/mmol. Radiochemical purity was reported to be 96%. This was diluted to a working solution in assay buffer (0.1 M phosphate-buffered saline, pH 7.2, with 0.1% gelatin, 0.01 M ethylenediaminetetra-acetic acid [EDTA], and 0.1% sodium azide added) to give 8,500 cpm/50 μLi, as determined with a Beckman LS-100C beta counter having 70% efficiency. Murine monoclonal anti-caffeine antibody, subclass IgG_2b (Peninsula Labs, Belmont, CA), purified by DEAE (diethylaminoethyl) column chromatography, was supplied in phosphate buffer at a concentration of 1 ng/ml. This was diluted 1:100 in assay buffer for use in the assay.

Caffeine USP standards (ICN Biochemicals, Costa Mesa, CA) were prepared gravimetrically in assay buffer. Separation of bound from

free caffeine was achieved with charcoal-coated dextran (0.625 g charcoal and 0.0625 g dextran T-70 per 100 ml of 0.1% gelatin in phosphate buffer).

Assay Procedure

The assay was performed in 12 x 75 mm borosilicate glass tubes. One ml saliva aliquots were centrifuged at 2500 RPM for 20 minutes to remove particulates, and the supernatant was saved for assay. A 1:100 dilution of supernatant with assay buffer was made of all samples and standards. One hundred microliters of diluted sample, 100 μl buffer, 50 μl tracer, and 50 μl antibody solution were added together and incubated at room temperature for 3 hours. Then 0.2 ml charcoal-coated dextran and 0.2 ml assay buffer were added and the tubes were centrifuged for 20 minutes at 2500 RPM. The supernatant (bound) was decanted into scintillation vials, and 10 ml scintillation cocktail (Aquasol-2, NEN, Boston, MA) were added. The same control saliva, with a known caffeine concentration of 0.74 μg/ml, was placed in the assay every 20 tubes, and standard curves were placed at the beginning, middle, and end of the assay. Two assays were required for the analysis of all the samples. Calculation of the unknown values was done with an unweighted log-logit RIA program (Chang, Rubin, and Yu, 1975). The assays were performed by S. S. McGeoy without his knowledge of subject treatment.

Results

Under our assay conditions, the monoclonal antibody bound 55 $\pm$ 5 % of the tracer in a caffeine-free sample. Concentrations of standard were 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 μg/ml. The standard curve was linear on log/logit plot, with an ED_{50} (the dose

producing 50% displacement of the tracer from the antibody) of 1.5 μg/ml. Assay sensitivity was 0.02 μg/ml, calculated as two standard deviations from the "zero" standard. The intra-assay coefficient of variation (CV) of 10 control serum replicates was < 5% at a value of 0.74 μg/ml, and the inter-assay CV of two assays was < 6% for the entire standard curve. Antibody cross-reactivity with theophylline was < 2%. To determine recovery of caffeine, 10 saliva samples representing a range of caffeine concentrations were re-assayed after being spiked with a known amount of caffeine USP prior to the initial centrifugation to remove particulates. Recoveries ranged from 96% to 102%.

The individual saliva caffeine concentrations in the four groups of subjects 40 minutes after the first dose of caffeine are plotted in Figure 1. After the first caffeine dose, the mean salivary caffeine concentrations were zero, 1.06, 2.00, and 3.68 μg/ml for the placebo, 150 mg, 300 mg, and 400 mg groups, respectively. Thus, the subjects given 300 mg had a mean salivary caffeine concentration 1.9 times that of the subjects given 150 mg, and the subjects given 400 mg had a mean concentration 3.5 times that of the subjects given 150 mg. The correlation coefficient between caffeine dose and saliva level for this set of measurements was 0.44 ($df=20$; $p<.025$, one-tailed). (Data from the subjects receiving placebo were not included in this calculation.)

The mean saliva caffeine concentrations in the four groups of subjects across the course of the study are shown in Figure 2. The caffeine administration times are indicated by the arrows below the abscissa. The placebo group had unmeasurable salivary caffeine throughout the study.

The 400 mg group received one dose of caffeine per 24 hours (first and fifth arrows in Figure 2). Saliva concentrations in this group 40 minutes after ingestion of the first dose were

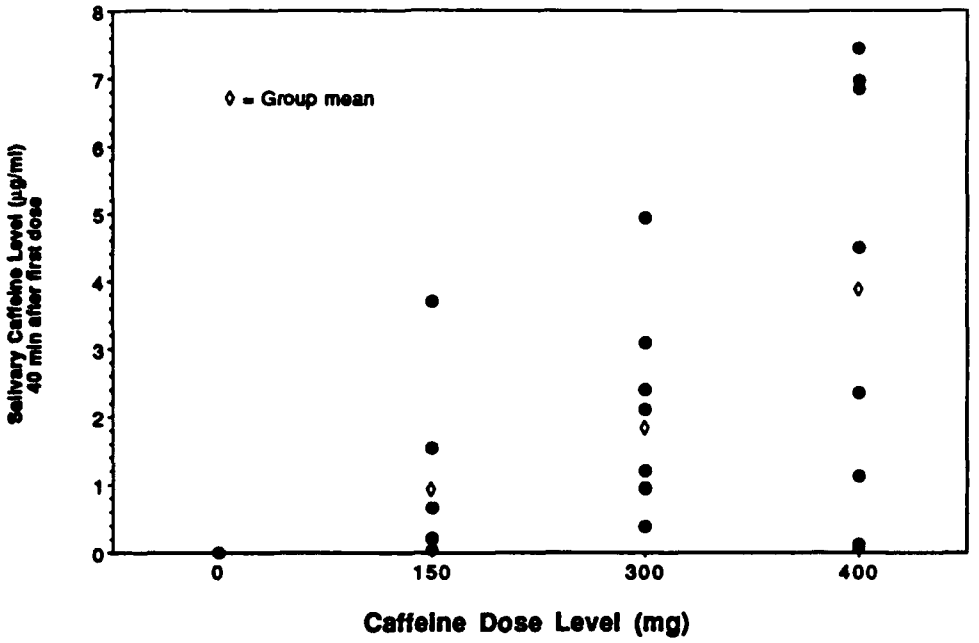

Figure 1. Individual salivary caffeine levels 40 minutes after the first dose, shown for each of the drug groups.

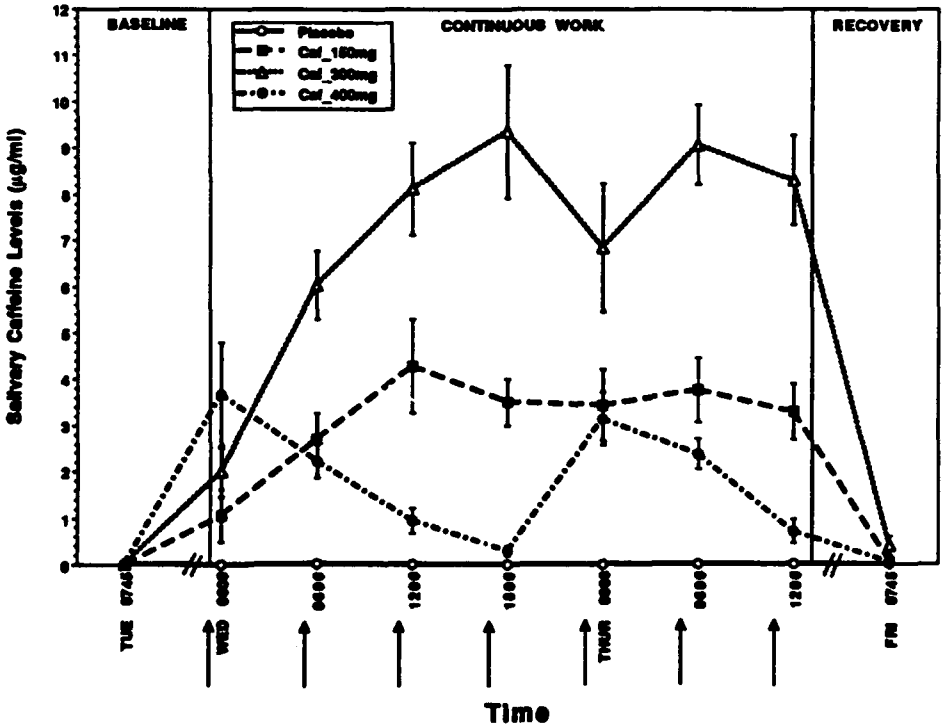

Figure 2. Salivary caffeine levels before, during and after repeated administration of caffiene.

approximately halved after six hours, 12 hours, and 18 hours. A similar pattern was seen after the dose administered the next night. The 150 and 300 mg groups, which received medication every six hours, showed a gradual increase in salivary caffeine which rose to a plateau after three doses. The plateau concentration (average of the fourth through the eighth saliva values) of the300mg group (8.3 μg/ml) was about twice that of the 150 mg group (3.7 μg/ml). ANOVA of the 300 mg and 150 mg group caffeine levels during the drug administration period showed a highly significant difference between the groups ($F(1,13)=14.003$, $p<0.003$). Only small amounts of residual caffeine were detectable in the samples taken 20 hours after the last dose. There was no significant correlation between saliva pH and the expected amount of salivary caffeine based on the dose-response relationship (under the assumption of consistent percent of caffeine entering the blood across subjects).

Discussion

The group mean caffeine concentrations measured in saliva in this study follow closely those that would be expected in plasma with the administration schedules used. Zylber-Katz et al. (1984), using high performance liquid chromatography (HPLC), found a linear relationship ($r = 0.98$) between plasma and salivary caffeine concentrations after a single dose of caffeine in 12 normal subjects. The correlation between dose and saliva level among our subjects 40 minutes after administration of the first dose is high (0.44). However, the broad distribution of saliva caffeine concentrations in all of the drug groups (Figure 1) would preclude using this assay as a measure of dose under these circumstances.

The correlation probably would have been higher and the distributions narrower if we could have sampled for a longer time

after administration. It usually takes about an hour to achieve maximal blood levels after an oral dose of caffeine on an empty stomach (James, 1991). The previously reported high correlations have generally involved delays of greater than one hour after administration (e.g.: 1.5 hours, Zylber-Katz et al., 1984; 4 - 6 hours, Khanna et al., 1982). Also, some of our subjects may have had delayed absorption; for example, two of the subjects who received 400 mg caffeine showed very low levels 40 minutes after the first dose (0.07 and 0.12 μg/ml; Figure 1). These subjects showed higher levels in the saliva sample taken about seven hours after administration (1.42 and 2.05 μg/ml, respectively), when significant clearance already would have occurred. This dose was administered about 3.5 hours after the subjects ate dinner. These subjects may have had slow gastric emptying, food having delayed absorption of the caffeine. Also, previous research has suggested that higher doses of caffeine, such as the 400 mg dose used herein, may take about twice as long to achieve maximum blood concentrations compared to lower doses (James, 1991). However, given that blood levels were not measured, we cannot rule out the possibility that these low levels specifically, and the broad intragroup variation generally, relate to interindividual variability in diffusion of caffeine from blood to saliva, rather than individual differences in absorption from the gut.

The timing of accumulation and clearance of caffeine in our subjects (Figure 2) is consistent with the serum half-life of three to seven hours that has been reported previously (Cook et al., 1976; Zylber-Katz et al., 1984; Smits, Thien, and van't Laar, 1985; Benet and Williams, 1990). Our salivary RIA thus provides a sensitive and specific, non-invasive method for determining caffeine concentrations in human subjects. While salivary caffeine levels soon after ingestion of a single dose may not be a reliable

measure of the dose taken, levels several hours after administration might well be. With more chronic administration, salivary levels appear to be a good dose measure. For example, salivary caffeine levels might be useful to estimate consumption in chronic caffeine users.

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